SUPPLEMENTAL MATERIALS

Methods: Eligibility

Eligible patients were at least 18 years of age, in first or second relapse of histologically confirmed glioblastoma following standard therapy (maximum feasible resection or biopsy, radiation and temozolomide), and had a Karnofsky Performance Status of at least 70 as well as adequate hematologic, renal and hepatic function. Patients who previously received radiation and temozolomide for low-grade glioma were permitted upon diagnosis of transformed glioblastoma. Patients were excluded for: prior receipt of PD-1/PD-L1 or VEGF/VEGFR targeting agents; grade >1 hemorrhage on baseline MRI; residual grade ≥ 2 chemotherapy or radiation-related toxicities (except alopecia and hematologic toxicity); salvage surgery within four weeks, radiation within 3 months and chemotherapy within 4 weeks (6 weeks for nitrosoureas) of enrollment; requirement of > 4 mg/day of dexamethasone; pregnancy or lactating; or active infection, autoimmune disease, or thromboembolism within 12 months.

Study Design and Treatment

This phase 2, multicenter, open-label, two-cohort study randomized recurrent, bevacizumab-naïve, glioblastoma patients to receive pembrolizumab (200 mg IV every 3 weeks) in combination with bevacizumab (10 mg/kg biweekly; cohort A; n=50) or pembrolizumab monotherapy (200 mg IV every 3 weeks; Cohort B; n=30). Cohort A included a 3+3 safety lead-in to assess the safety of combination therapy as these two agents had not been previously combined for glioblastoma patients. Each cycle was 42 days and required an ANC ≥ 1,000/μl, platelet count ≥ 100,000/μl, serum creatinine ≤ 1.5 times upper limit of normal (X ULN), SGOT and bilirubin ≤ 2.5 X ULN, urine protein ≤ 30 mg/dL and resolution of any grade ≥ 3 toxicity at least possibly related to prior therapy to grade ≤1 or pretreatment baseline. Allowed supportive

medications included anti-emetics, anti-diarrheal agents, hematopoietic growth factors and low molecular weight heparin. Dexamethasone was also allowed for symptomatic cerebral edema, but investigators were encouraged to use the lowest dose for as short as possible. Other cancer therapies were not permitted. No dose reductions were included for bevacizumab. Pembrolizumab dose modification for toxicity included an increase of the dosing interval to 4 (dose level -1) and 6 weeks (dose level -2). The trial protocol provided criteria for permanent discontinuation of either study agent. For cohort A, if one study agent was discontinued for toxicity, the other agent was allowed to continue provided it was unrelated to the toxicity. Treatment continued until tumor progression, unacceptable toxicity, non-compliance or withdrawal of consent.

Toxicity was monitored and graded using Common Terminology Criteria for Adverse Events (CTCAE, version 4.0). Safety assessments included weekly physical examination, vital signs, and routine hematology, blood chemistry, and urinalysis during study therapy.

Biomarker Analyses

An aliquot of the most recent tumor sample available prior to initiation of study therapy, including either archival tumor from original diagnosis or tumor obtained upon recurrence was requested for all patients. For each patient, a minimum of one formalin-fixed paraffin-embedded (FFPE) archival tumor tissue block or a minimum of 10 FFPE unstained sections were submitted to DFCI. PD-L1 expression and TIL density were performed by a board-certified pathologist at a commercial vendor (QualTek, Molecular Laboratories, Santa Barbara, CA). PD-L1 expression was assessed using an established immunohistochemistry assay[1] and defined as positive if $\geq 1\%$ of cells exhibited membranous staining. Density of TILs was objectively determined as previously described by a board-certified pathologist from a hematoxylin and eosin stained

tumor section based on the following scale which included evaluation of an average of 3-5 high-power (20X) fields (HPF): 0 = <1 TIL per HPF: 1 = 1-10 TIL per HPF; 2 = 11-20 TIL per HPF; and 3 = >20 TIL per HPF.[2] Immune activation gene expression panel (GEP) was assessed by nanostring by Merck & Co., Inc., Kenilworth, NJ, USA as previously described.[3,4] Plasma biomarkers were evaluated in both cohorts as previously described[5] at baseline and on study therapy at day 1 of cycle 3 (after 84 days/12 weeks). Plasma was separated from fresh blood samples, and all samples were aliquoted and frozen. Analysis was carried out for biomarkers of angiogenesis and inflammation molecules at the end of the study including: VEGF, placental growth factor (PlGF), VEGF-C, VEGF-D, soluble (s)VEGFR1, basic fibroblast growth factor (bFGF), and sTie-2 using multiplex arrays from Meso-Scale Discovery (Gaithersburg, MD); and ANG-2 using enzyme-linked immunosorbent assay (ELISA) plates from R&D Systems (Minneapolis, MN). All samples were run in duplicate in the CLIA-certified core of the Steele Laboratories at Massachusetts General Hospital.

Statistical Analyses

The primary endpoint for each cohort was progression-free survival at 6 months on the intent-to-treat population using RANO.[6] Secondary endpoints were determination of objective response rate (ORR), OS and overall safety and tolerability. Exploratory endpoints included association of outcome with archival tumor PD-L1 expression, TIL density and immune activation signature, levels of circulating cytokines as well as changes in patient neurologic function using the NANO scale.[7]

Following completion of the safety lead-in for cohort A, each cohort accrued using a single-stage design. Time to event analyses for PFS and OS were calculated using the Kaplan-Meier method from time of treatment start to date of progression or death. Patients who did not

progress or die within 28 days of treatment end were censored at the date of last assessment. The Log-Rank test was used for comparison between groups.

With accrual of 50 patients, study therapy for cohort A was to be considered promising if 30 or more patients were progression-free at 6 months. The design discriminates between true PFS-6 ≤ 40% and >60%, with type I error rate of 5% and 87% power. A PFS-6 rate of 40% was chosen based on phase II data for bevacizumab in the same target population.[8,9]

For cohort B, with accrual of 30 patients, study therapy was to be considered promising if ≥ 10 patients were progression-free at 6 months. The design discriminates between true PFS-6 ≤ 10% and > 30%, with type I error rate of 5% and 84% power. A 10% PFS-6 rate was chosen based on meta-analysis data treated with salvage therapy, excluding anti-angiogenic therapy, for the same target population.[10]

Changes in neurologic function, including specific domains and overall score, were assessed over the course of study therapy relative to pretreatment baseline using the NANO scale until patients came off study.[7] Scores while on study therapy prior to progression were compared to the baseline score using R (version 3.4.3). NANO response and progression were assessed for correlation with RANO progression, KPS score, corticosteroid use and corticosteroid dose increase prior to initiation of cycle 3 using Fisher's Exact Test. Reduced availability of data on these factors at later time points limited additional analyses.

The relationship between archival paraffin-embedded and formalin-fixed tumor immunocorrelative biomarkers including PD-L1 expression, TIL density and immune activation signature with PFS or OS were analyzed by fitting a univariate Cox Proportional Hazard model with results presented as hazard ratios (HRs). HRs represented the increase/decrease in the risk of progression or death per unit increase in the biomarker tested. Change in the blood biomarkers

from before and during treatment was summarized using descriptive statistics, and differences between time points were evaluated using the Wilcoxon signed rank test. Relationship between circulating biomarker levels at baseline and on treatment and PFS and OS were analyzed by fitting a univariate Cox Proportional Hazard model and the results are presented with hazard ratios (HRs).

SUPPLEMENTAL TABLES

Supplemental Table 1. Baseline and posttreatment plasma biomarker concentration changes by treatment cohort.

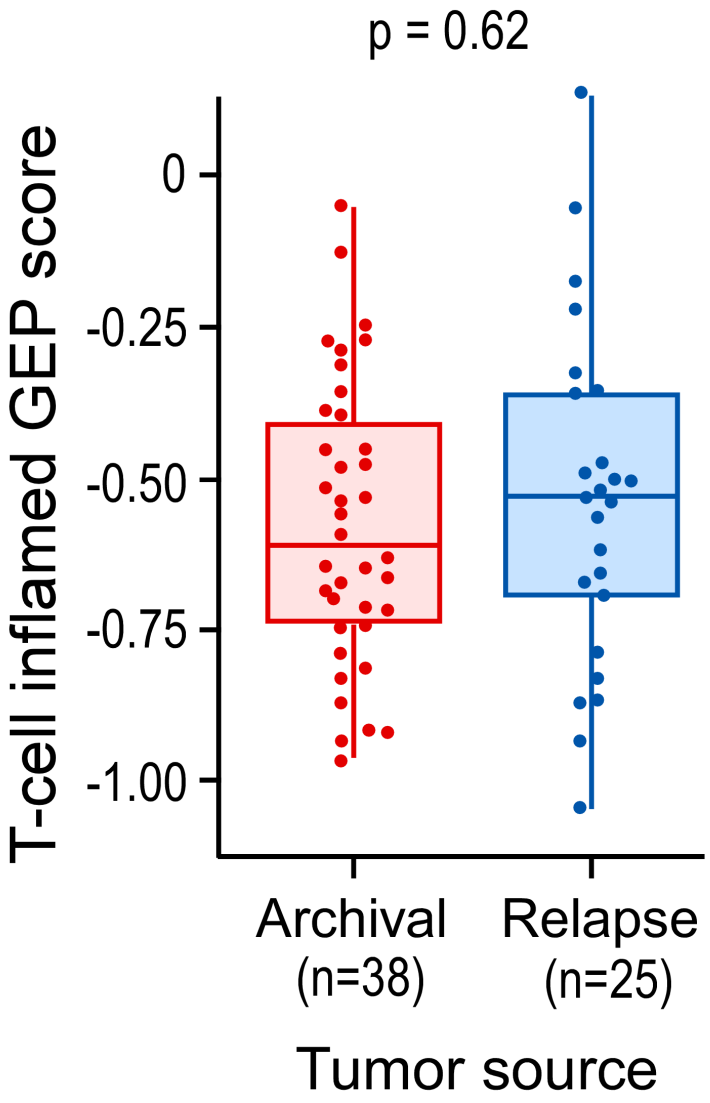
Biomarker (pg/ml)	Cohort A		Cohort B	
	Baseline (n=33)	**Day 84 (n=18)**	**Baseline (n=19)**	**Day 84 (n=5)**
Ang-2	2,145 (1,641, 2,422)	1,537 (1,217, 2,561)	1,838 (1,546, 2,481)	2,328 (1,539, 3,428)
P value	0.00037		0.88	
sVEGFR1	65.8 (56.9, 78.0)	66.8 (56.8, 90.8)	66.5 (57.2, 86.3)	77.0 (66.4, 82.8)
P value	0.90		0.88	
PlGF	8.6 (7.8, 9.5)	17.6 (14.0, 22.1)	9.1 (7.6, 10.3)	9.0 (6.8, 9.5)
P value	0.00012		1.0	
sTIE2	4,255 (3,814, 5,124)	4,746 (3,843, 5,439)	4,657 (3,835, 4,888)	4,641 (3,859, 5,013)
P value	0.67		0.88	
VEGF	106.9 (81.1, 128.8)	49.0 (49.0, 57.5)	81.6 (59.1, 97.2)	74.6 (68.7, 75.4)
P value	0.00012		0.88	
VEGF-C	59.2 (57.0, 96.2)	66.5 (57.0, 80.1)	57.0 (57.0, 74.2)	57.0 (57.0, 57.0)
P value	0.91		1.0	

Supplemental Table 2. Correlation between circulating biomarker levels at baseline and overall survival (OS) and progression-free survival (PFS) by treatment cohort.

	Baseline				Post-Treatment (Day 84)			
Biomarker	**Cohort A – P+B**		**Cohort B – P alone**		**Cohort A – P+B**		**Cohort B – P alone**	
	OS	**PFS**	**OS**	**PFS**	**OS**	**PFS**	**OS**	**PFS**
Ang-2	1.00	1.00	1.000	1.001	1.000	1.00	0.999	1.000
P value	0.46	0.18	0.56	0.11	0.13	0.10	0.24	0.83
sVEGFR1	0.99	1.01	1.008	1.007	0.998	1.017	0.993	0.995
P value	0.31	0.33	0.038	0.082	0.75	0.0090	0.39	0.36
PlGF	1.14	1.13	1.571	1.092	1.022	0.994	1.142	2.602
P value	0.30	0.30	0.0031	0.45	0.76	0.94	0.60	0.18
sTIE2	1.00	1.00	1.000	1.000	1.000	1.000	0.999	1.000
P value	0.25	0.44	0.14	0.53	0.56	0.35	0.17	0.44
VEGF	1.005	1.002	1.000	1.002	1.072	1.06	0.988	0.993
P value	0.08	0.40	0.96	0.65	0.026	0.081	0.64	0.42
VEGF-C	1.0	0.99	0.995	0.999	1.003	1.005	0.884	0.884
P value	0.99	0.91	0.50	0.90	0.74	0.61	0.99	0.99

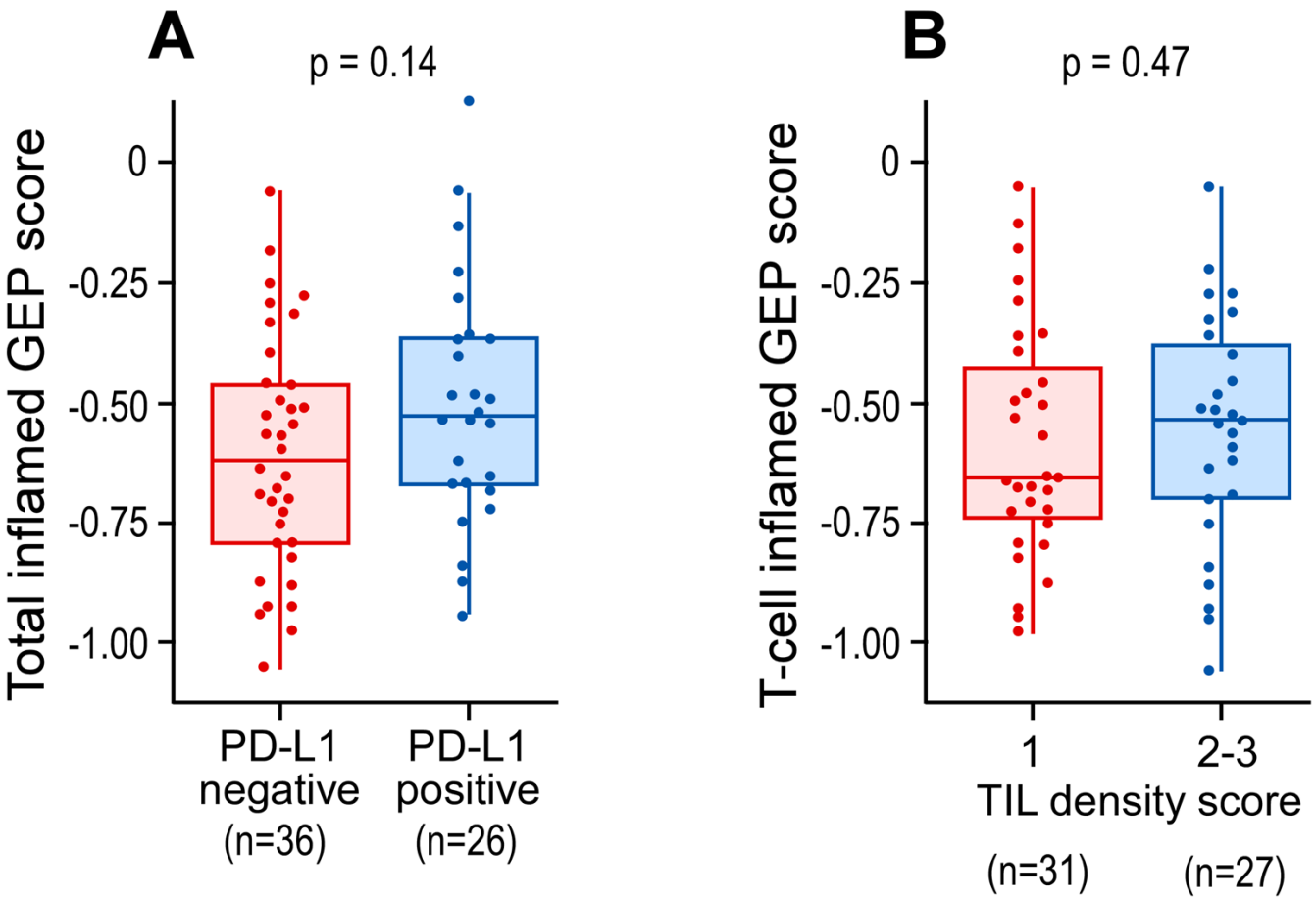
SUPPLEMENTAL FIGURES

Supplemental Figure 1.



Supplemental Figure 1 (online only). Tumor T cell immune activation GEP score for tumors obtained at original diagnostic surgery (archival) versus at relapse prior to start of study therapy.

Supplemental Figure 2



Supplemental Figure 2 (online only). Tumor T cell immune activation gene expression profile (GEP) relative to PD-L1 expression (A) and TIL density score (B).

SUPPLEMENTAL MATERIALS: REFERENCES

1 Dolled-Filhart, M. *et al.* Development of a Prototype Immunohistochemistry Assay to Measure Programmed Death Ligand-1 Expression in Tumor Tissue. *Arch Pathol Lab Med* **140**, 1259-1266, doi:10.5858/arpa.2015-0544-OA (2016).

2 Habra, M. A. *et al.* Phase II clinical trial of pembrolizumab efficacy and safety in advanced adrenocortical carcinoma. *J Immunother Cancer* **7**, 253, doi:10.1186/s40425-019-0722-x (2019).

3 Ayers, M. *et al.* IFN-gamma-related mRNA profile predicts clinical response to PD-1 blockade. *J Clin Invest* **127**, 2930-2940, doi:10.1172/JCI91190 (2017).

4 Ott, P. A. *et al.* T-Cell-Inflamed Gene-Expression Profile, Programmed Death Ligand 1 Expression, and Tumor Mutational Burden Predict Efficacy in Patients Treated With Pembrolizumab Across 20 Cancers: KEYNOTE-028. *J Clin Oncol* **37**, 318-327, doi:10.1200/JCO.2018.78.2276 (2019).

5 Reardon, D. A. *et al.* Phase 2 and biomarker study of trebananib, an angiopoietin-blocking peptibody, with and without bevacizumab for patients with recurrent glioblastoma. *Cancer* **124**, 1438-1448, doi:10.1002/cncr.31172 (2018).

6 Wen, P. Y. *et al.* Updated response assessment criteria for high-grade gliomas: response assessment in neuro-oncology working group. *J Clin Oncol* **28**, 1963-1972 (2010).

7 Nayak, L. *et al.* The Neurologic Assessment in Neuro-Oncology (NANO) scale: a tool to assess neurologic function for integration into the Response Assessment in Neuro-Oncology (RANO) criteria. *Neuro Oncol* **19**, 625-635, doi:10.1093/neuonc/nox029 (2017).

8 Kreisl, T. N. *et al.* Phase II trial of single-agent bevacizumab followed by bevacizumab plus irinotecan at tumor progression in recurrent glioblastoma. *J Clin Oncol* **27**, 740-745 (2009).

9 Friedman, H. S. *et al.* Bevacizumab alone and in combination with irinotecan in recurrent glioblastoma. *J Clin Oncol* **27**, 4733-4740 (2009).

10 Wu, W. *et al.* Joint NCCTG and NABTC prognostic factors analysis for high-grade recurrent glioma. *Neuro Oncol* **12**, 164-172, doi:nop019 [pii]
10.1093/neuonc/nop019 (2010).